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Bone regeneration potential of a soybean-based filler: experimental study in a rabbit cancellous bone defects

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Abstract Autologous and allogenic bone grafts are considered as materials of choice for bone reconstructive surgery, but limited availability, risks of transmittable diseases and inconsistent clinical performances have prompted the development of alternative biomaterials. The present work compares the bone regeneration potential of a soybean based bone filler (SB bone filler) in comparison to a commercial 50:50 poly(D,L lactide–glycolide)-based bone graft (Fisiograft® gel) when implanted into a critical size defect (6-mm diameter, 10-mm length) in rabbit distal femurs. The histomorphometric and microhardness analyses of femoral condyles 4, 8, 16 and 24 weeks after surgery showed that no significant difference was found in the percentage of both bone repair and bone in-growth in the external, medium and inner defect areas. The SB filler-treated defects showed significantly higher outer bone formation and microhardness results at 24 weeks than Fisiograft® gel ($P < 0.05$). Soybean-based biomaterials clearly promoted bone repair through a mechanism of action that is likely to involve both the scaffolding role of the biomaterial for osteoblasts and the induction of their differentiation.

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1 Introduction

A relatively rapid and complete bone repair around bone implants is an important factor to ensure the early implant stability as it minimizes undesired changes in the tissue structural and biomechanical features leading to bone rarefaction and microarchitectural deterioration [1, 2]. In particular, the treatment of critical size bone defects generated by either trauma or disease relies on the use of biomaterials able to support tissue regeneration [3, 4]. Indeed, when defects reach a critical size, bone is unable to regenerate spontaneously and bone fillers are required to guide its formation [1–3]. Mineralized and non-mineralized bone grafts which are derived from the same patient (autograft) and from human or animal donors (allograft) are considered the gold standard in surgery [4–7]. However, drawbacks are linked to their use; limited availability and patient's morbidity affect the use of autografts, while risks of transmittable diseases are associated to allografts [7]. Collagen, extracted from animal sources or of recombinant origin, has also been made available in form of films and sponges and used in surgery as an alternative to autografts and allografts. However, these biomaterials are affected by adverse reactions leading to fibrotic tissue formation [1]. Additional drawbacks are the risk of transmittable diseases linked to the use of extracted collagen, while recombinant products suffer of relatively high manufacture costs [1].

In the attempt to make available biomaterials with higher clinical performance and sustainable costs, synthetic bone fillers have been developed. The most used synthetic bone fillers are: (i) ceramics (hydroxyapatite, HA, tri-calcium phosphate, TCP, bioglasses) and (ii) poly(lactic/glycolic) acid (PLGA)-based hydrogels. HA, TCP, and bioglass, mainly delivered in forms of porous scaffolds and

granules, have excellent osteoconductive properties [8–11]. These biomaterials have been shown to support osteoblast adhesion and proliferation in vitro [12] and to establish a strong bonding with the newly deposited bone mineral phase in vivo [13]. However, their brittleness impairs handling and adaptation to the bone defect during surgery. In addition, the resorption rate of these ceramics cannot be finely tuned to the bone regeneration and remodeling rate [14]. PLGA-based hydrogels have also shown satisfactory bone regeneration potential [15]. Although these biomaterials completely biodegrade into CO_2 and water [16], it is known that the polymer fragments formed during their degradation elicit inflammatory response and bone resorption [17].

It is widely accepted by scientists and clinicians that a true bone induction cannot be obtained by these traditional bone fillers unless growth factors such as, for example, the bone morphogenetic protein 2 (BMP-2) are loaded in their structure [18]. Clinical studies have shown the potential of BMP-2-loaded bone fillers in accelerating bone regeneration [18], but relatively high amounts of this growth factor are required to achieve satisfactory clinical results. As a consequence, the costs of BMP-2-based bone fillers are inevitably increased and concerns arise about their potential carcinogenic effect [19].

Soybean is a natural material made of protein and carbohydrate fractions (approximately 40% by weight for each fraction), of an oil fraction (approximately 18%), and of minerals (approximately 2%) [20]. Soybean also contains isoflavones, phytoestrogens with an ascertained action on eukaryotic cells [20]. Isoflavones inhibit tumor cell proliferation and immunocompetent cell activation and seem to reduce scar formation in wound healing [21, 22]. Recently, a new class of biomaterials has been developed from defatted soybean curd and flour [23, 24]. The processing of these components by either thermosetting or extraction allows the preparation of materials with different physico-chemical properties; by these processes membranes, films, granules and gels can be obtained. The bone regeneration potential of these biomaterials has been demonstrated by in vitro studies highlighting their inhibitory effect on monocytes/macrophages and osteoclasts as well as their ability to induce osteoblast differentiation and bone noduli mineralization [25–27].

In this study we report the bone regeneration potential of two different filler formulations of soybean-based biomaterials. The study was performed in a rabbit bone critical size model and compared the bone regeneration of the soybean-based biomaterials with that of a commercial PLGA-based bone filler, the Fisiograft® gel.

2 Materials and methods

2.1 Synthesis of the soybean-based biomaterials

2.1.1 Hydrogel

Commercial soy flour, defatted by a standard hexane extraction procedure [23], was mixed with 80:20 ethanol–water at a solvent to flour ratio of 10:1. The suspension was shaken at a 45° angle, at 200 rpm, in a shaking incubator at 30°C, for 4 h and then allowed to cool and settle. The supernatant was then removed and centrifuged at 2500 rpm for 10 min to remove any suspended solids. The suspension was sequentially filtered through a $1'' \times 24''$ glass column, packed with 2 cm glass wool, 1 cm silica gel, and 1 cm glass wool and through a (pre-washed) Whatman filter paper. The collected filtered solvent fraction was then rotary evaporated at 30°C, with high vacuum leaving small volume of extract in water. The extract was then freeze-dried for 72 h to produce a dry powder. The dry extract was weighed out (320 mg) into a 15 ml glass vial and reconstituted in 96 µl of 0.1 M $CaCl_2$. The obtained hydrogel was left to hydrate at 37°C for 24 h before mixing with soybean granules.

2.1.2 Granules

Defatted commercial soy flour was processed into curd by a standard procedure [24], the curd cut into slices (0.5 cm thick) and thermoset at 60°C for 24 h. The cooled thermoset material was then ground and the granules sieved using a shaking sieve tower. The granules of size 212–300 µm were collected for biomaterial use. The granules were freeze dried for 24 h to remove any residual water content. The granule size was selected to match that of biomaterials (e.g. HA granules and moreslized allograft particles) currently used in clinics.

2.1.3 Fillers

Two different formulations were made available for in vivo testing. The first formulation was obtained by adding an equivalent weight of tofu granules (212–300 µm) to the hydrogel to give a 50:50 gel-to-granule formulation (soybean based—SB bone filler). The gel/granule paste was then loaded into a 1 ml plastic syringe and the end capped. To avoid separation between the granule and the gel phase during injection, the lower end of the syringe was cut and subsequently sealed with a plastic cap prior to material preparation and sterilisation. The loaded syringe was then sealed in a single sterilisation pouch and sterilised with gamma irradiation (25 kGy).

The alternative SB bone filler formulation was provided in a three-component kit that included (i) SB powder to be reconstituted into hydrogel, (ii) SB granules (212–300 μm) and (iii) 0.1 M $CaCl_2$ solution. The three-component containing vials were then sealed in a single sterilization pouch and sterilized with gamma irradiation (25 kGy). This three-component filler was prepared aseptically by the surgeon to the required consistency immediately prior to implantation. In particular, 300 mg hydrogel powder was mixed with 150 mg granules and 100 μl of 0.1 M $CaCl_2$ [25].

The PLA/PGA (50:50) copolymer Fisiograft® gel (Ghimas S.p.A, Casalecchio di Reno—Bologna, Italy) was used as a control material. Fisiograft® gel has been originally designed for use in bone grafts as a space filler for guided tissue regeneration or guided bone regeneration for oral surgery [28, 29].

2.2 In vivo experiments

The study was performed in compliance with European and Italian Law on animal experimentation: the animal experimental protocol was received from and approved by the Ethical Committee of Rizzoli Orthopaedic Institute and the Italian Ministry of Health.

Twenty-four adult male New Zealand rabbits (Charles River Laboratories Italia S.r.l., Calco - Lecco, Italy), 3.250 ± 0.350 kg body weight, were chosen as a model for studying the biomaterial osteo-regenerative potential in cancellous bone. General anaesthesia was induced with an intra-muscular injection of 44 mg/kg ketamine (Ketavet 100, Farmaceutici Gellini SpA, Aprilia Lt, Italy) and 3 mg/kg xylazine (Rompun Bayer AG, Leverkusen, Germany), and assisted ventilation with O_2/N_2O (1/0.4 l/min) and 2–2.5% isofluorane (Forane, Abbott SpA, Campoverde di Aprilia—Latina, Italy).

Critical size defects (6-mm diameter, 10-mm length) were transversally created in the femoral distal epiphysis of both posterior limbs by a standardized surgical procedure. A 2-cm skin incision was made on the lateral aspect of the distal femoral condyle. Bilateral confined cancellous defects were stepwise drilled in both limbs with a 3.2-mm drill. The defects were subsequently expanded with a 6.0-mm drill. The depth of the defects was 10 ± 0.5 mm as measured by a digital caliper. In a first experimental step, the defects generated in two rabbits were left untreated (untreated-group), while eighteen rabbits were operated to both femurs that were treated with the SB bone filler formulation consisting of the gel/granule paste. In a second experimental step, four rabbits were treated with the three-component SB bone filler formulation (Table 1). In both experimental steps, the counter-lateral femoral defect was treated with Fisiograft® gel (Table 1). The soft tissues were sutured in two layers with Dexon 3-0 and silk 3-0. Antibiotic therapy (enfloroxacin, 100 mg/kg—Baytril, Bayer, Milan, Italy) was administered preoperatively and for 5 days after surgery. Analgesics (metamizole chloride, 50 mg/kg—Farmolisina, Vetem SpA, Porto Empedocle—Grosseto, Italy) were prescribed in the immediate postoperative period. The animals were accommodated in the animal house at similar conditions to minimize differences in loading/gait pattern in each animal and between animals. However, no specific measurement of these parameters was performed.

On days 10, 9, 2 and 1 prior to killing, the animals received an intra-muscular injection of oxytetracycline (30 mg/kg). Four ($n = 6$), 8 ($n = 8$), 16 ($n = 2$), and 24 ($n = 7$) weeks after surgery, the animals were killed by pharmacological euthanasia under general anesthesia with intravenous administration of Tanax (Hoechst, Frankfurt am Main, Germany). Retrieved femoral condyles, stripped of soft tissues, were fixed in 4% (v/v) buffered paraformaldehyde and prepared for histology.

2.3 Histomorphometry

The femoral condyles were dehydrated in graded series of alcohols/water mixture followed by complete dehydration in absolute alcohol. Following dehydration the specimens were embedded in poly(methyl methacrylate) resin. Blocks were sectioned along a plane perpendicular to the bone

Table 1 Experimental set-up

Fillers	No. rabbits	Experimental times			
		4 weeks	8 weeks	16 weeks	24 weeks
First experiment					
SB bone filler (left condyle)/Fisiograft® gel (right condyle)	6	6/6 sites			
	6		6/6 sites		
	5				5/5 sites
Second experiment					
3-component bone filler	2		2/2 sites		
	2			2/2 sites	

surface and a series of sections of 200 ± 10 μm in thickness, spaced 300 μm apart (because of the thickness of the microtome diamond saw), were obtained with a Leica 1600 diamond saw microtome (Leica SpA, Milan, Italy). Finally, sections were grounded to a thickness of 30 ± 10 μm and stained with Acid Fuchsine and Fast Green or with Von Kossa.

Histomorphometric analysis was performed on sections derived from the epiphysis trabecular bone and excluded the cortical bone and the defect distal end. A total of three sections were analyzed for each defect by means of histological and histomorphometric studies. This analysis started at 2 mm from the external border of the defect to 2 mm of the internal defect size. Light (LM) and polarised-light microscopy (PLM) analyses were performed using an optic microscope (BX41, Olympus Italia S.r.l., Segrate—Milano, Italy) connected to an image analysis system (Qwin, Leica Imaging Systems Ltd, UK). The analysis focused on the evaluation of the trabecular invasion into the defect and on the quality of the newly formed bone. Using the digitalized images obtained from the transverse overview of the defect (×1.25 magnification of the whole 6 mm diameter defect), the following parameters were determined within the region of interest (ROI):

(a) Trabecular invasion was assessed by a semi-quantitative scoring system based on a 1–5 scale comparing the counter-lateral defects of each animal. The scoring system referred to 5: healing of bone-defect, 4: presence of bone remodeling inside the bone-defect area, 3: dense trabeculae invading bone-defect, 2: bone regeneration starting from the bone-defect edges with very thin trabeculae, 1: no bone-defect invasion.
(b) Bone healing rate (%) was quantitatively evaluated as trabecular invasion percentage that is calculated as the percentage of the bone invasion of the defect divided by the initial area of the defect.
(c) Bone area surface (%) was evaluated in three different regions to assess the bone distribution. The ROI was divided into three circles (inner, middle and outer), each with a diameter of 1 mm. The bone area in these three separate fields was expressed as a percentage of the area of each circle [30].

Finally, the nomenclature and methodology approved by the American Society of Bone and Mineral Research (ASBMR) was followed to measure newly grown bone quality as mineral apposition rate (MAR) and bone formation rate (BFR/B.Pm) [31]. The injection of oxytetracycline to the animals allowed the assessment of both MAR and BFR/B.Pm by epifluorescent microscopy. In particular, MAR was measured as the distance between the midpoints of two consecutive deposited and epifluorescent fronts of oxytetracycline divided by the time between the midpoints of the labeling periods. BFR/B.Pm values were obtained by multiplying the MAR value by the sum of 1/2 single label perimeter and double label perimeter. Bone quality of the newly formed bone inside the defect (when present) was compared with that of bone at the same distal femur area of control rabbits of the same race, age and weight killed after the same period of time.

2.4 Bone microhardness

The resin-embedded blocks for histology were used to measure bone hardness 4, 8 and 24 weeks after surgery by means of an indentation test (Microhardness VMHT 30, Leica, Wien, Austria) [32, 33]. The microhardness measurements were taken tangentially to the interface with a Vickers indenter applied to the bone at a load of 0.05 kg and dwell time of 5 s. The average value for each sample was calculated from a mean of 10 measurements for each examined area at two sites: (i) a site within 200 μm from the interface and outside the defect in the pre-existing host bone, (ii) a site at 3000 μm from it. Finally, the bone maturation index (BMI) was calculated by dividing the microhardness of the bone re-grown at the interface (HV200 μm) by the microhardness of the pre-existing bone (HV1000 μm) multiplied by 100.

2.5 Statistical analysis

Statistical evaluation of data was performed using the software package SPSS/PC + StatisticsTM 12.1 (SPSS Inc., Chicago, IL USA). Data are reported as median (min–max) and considered significantly different at $P < 0.05$. Levene's test showed a non-parametric distribution of the histomorphometric data between SB bone filler and Fisiograft® gel. Therefore, these data were analysed by the non-parametric Wilcoxon signed-rank test, followed by the Monte Carlo methods to compute one-sided probability. No statistical comparisons were done with the 3-component SB bone fillers due to the limited number of specimens.

3 Results

All the animals survived until the final experimental time and no operative or post-operative complications were encountered. Before bone sample retrieval, the clinical and macroscopic evaluation revealed that neither necrosis nor signs of infection were observed.

As expected, the formation of new bone in untreated cavities remained restricted to the edge of the defects and the largest part of the center of the defect remained free of

bone up to 24 weeks (data not shown). A semi-quantitative assessment of the bone in-growth promoted at 4 weeks by injectable SB bone filler and Fisiograft® gel showed a better performance of the PLGA-based biomaterial (Table 2, Fig. 1a–f). In the case of SB bone filler, a dense network of relatively small and convolute trabeculae was always found at the periphery of the surgical defect and in close proximity of the biomaterial granules (Fig. 1a, c, e). The degree of bone in-growth appeared to depend on the granule packing within the defect; the SB bone filler appeared to be too densely packed into the defect in two out of six implants (Fig. 1e) thus preventing any significant bone in-growth. In the case of Fisiograft® gel, bone trabeculae significantly invaded the tissue defect in the majority of the cases although at different degrees (Fig. 1b, d, f). Fisiograft® gel showed trabecular invasion higher than SB bone filler in four out of six cases; in two cases bone completely invading the defect.

At week 8, different degrees of bone in-growth were observed within the SB inter-granular space in five out of

Table 2 Semi quantitative histological scoring of bone-defects filled with SB bone filler and Fisiograft® gel comparing the counter-lateral defects of each animals

Fillers: Experimental time	SB bone filler	Fisiograft® gel
4 weeks	2 (1–3)	3 (1–5)
8 weeks	4 (1–4)	2 (1–3)
24 weeks	3 (1–5)	3 (1–5)

Median (min–max)

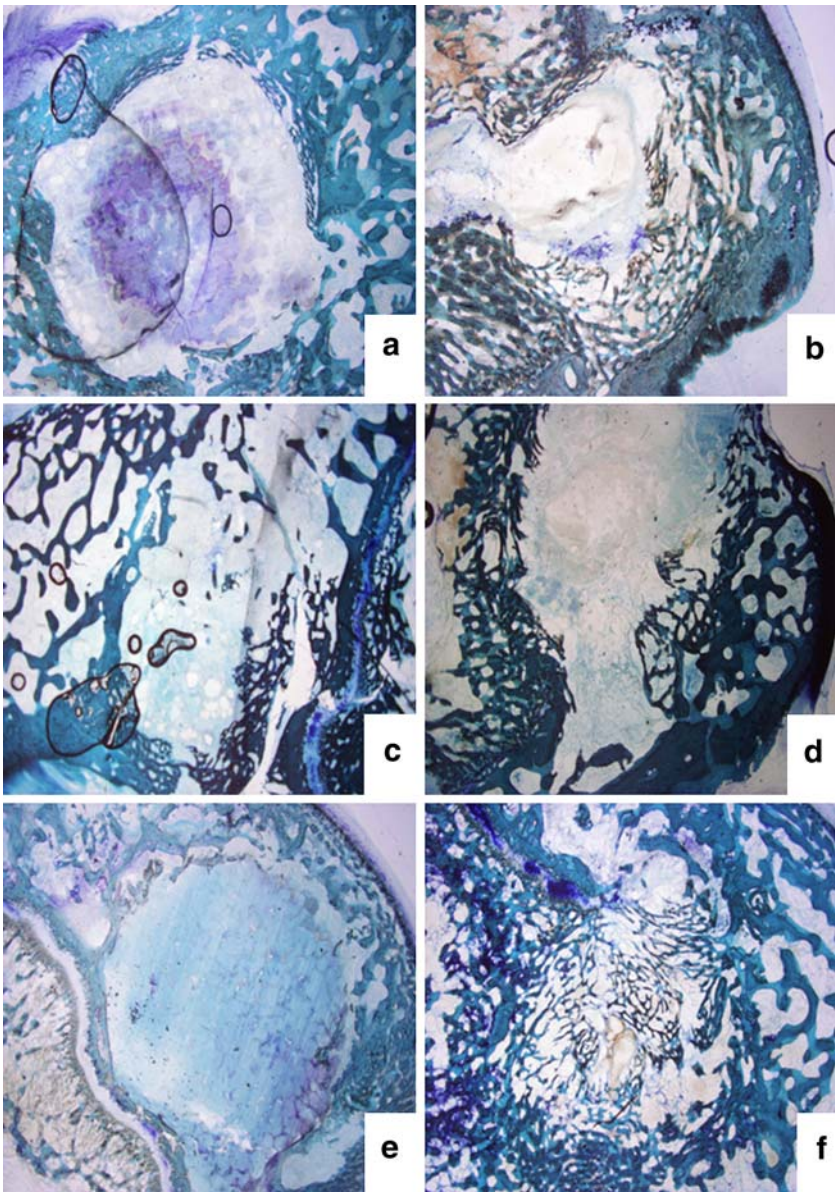

Fig. 1 Typical histological pattern of bone repair in defects treated with SB bone filler (**a**, **c**, **e**) and Fisiograft® (**b**, **d**, **f**) after 4 weeks of implantation. Images show a direct comparison (**a** vs. **b**, **c** vs. **d**, **e** vs. **f**) of the bone repair performance of the two biomaterials when implanted in contra-lateral femurs. Images were selected to show different degrees of bone in-growth and biomaterials packing and degradation. Photos were taken at ×1.25 magnification

six cases (Table 2, Fig. 2a, c, e). As for the implants retrieved after 4 weeks, bone in-growth was impaired only in cases where SB granules were excessively packed (data not shown). Although is some cases the trabecular bone was still characterized by small and convolute trabeculae (Fig. 2c), in most of the SB implants, relatively larger and more mature trabeculae were observed (Fig. 2a, e). The central part of the SB-treated defects showed areas of inflammatory response (Fig. 2a, c, e, intense central staining (blue in the original micrographs)). At this experimental point, Fisiograft® gel showed poor trabecular invasion and histological features typical of bone resorption (Fig. 2b, d, f). The histological features were confirmed by a LM and PLM analysis at higher magnification (Fig. 3a–f). This more detailed analysis showed the progressive in-growth of the trabecular bone throughout the SB granules and the close apposition of newly calcifying trabeculae on their surface (Fig. 3a, c). Conversely, Fisiograft®-treated defects showed areas of physiological bone formation at 4 weeks of implantation (Fig. 2b) followed by bone resorption around the degrading material after 8 weeks (Fig. 3d, f). Overall, SB bone filler promoted a level of bone in-growth higher than Fisiograft® gel in four out of six cases.

At 24 weeks, both the biomaterials scored for satisfactory trabecular invasion in the majority of the cases (Fig. 4a–f). In two cases excessive SB granule packing seemed to lead to limited bone regeneration and in one case to fibrosis (Fig. 4e, arrowhead). No fibrotic capsule was found in the SB filler-treated defects at shorter implantation

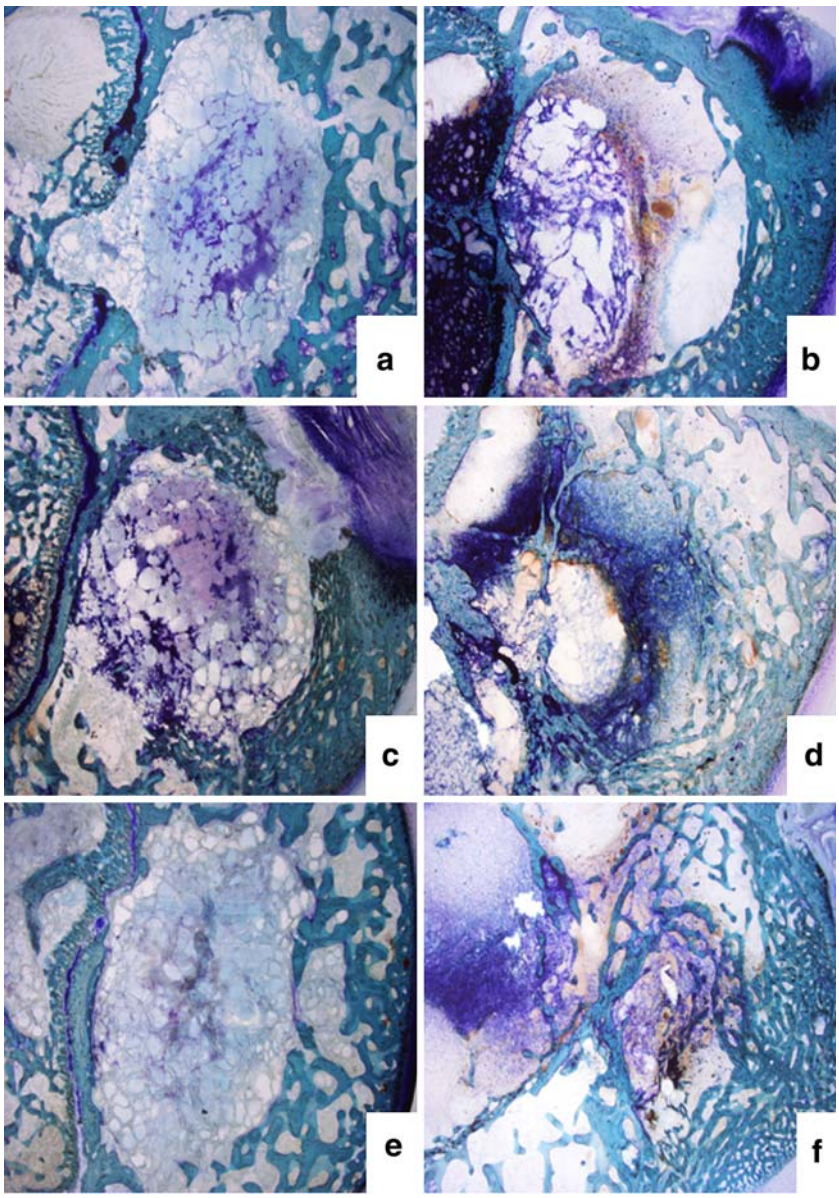

Fig. 2 Typical histological pattern of bone repair in defects treated with SB bone filler (**a**, **c**, e) and Fisiograft® (**b**, **d**, **f**) after 8 weeks of implantation. Images show a direct comparison (**a** vs. **b**, **c** vs. **d**, **e** vs. **f**) of the bone repair performance of the two biomaterials when implanted in contra-lateral femurs. Images were selected to show different degrees of bone in-growth and biomaterials packing and degradation. Intense staining (blue in the original micrographs) in the bone defects indicates area of inflammatory response. Light-stained (brown color in the original micrographs) areas indicate an acidic environment typical of PLA/PGA degradation. Photos were taken at ×1.25 magnification. (Color figure online)

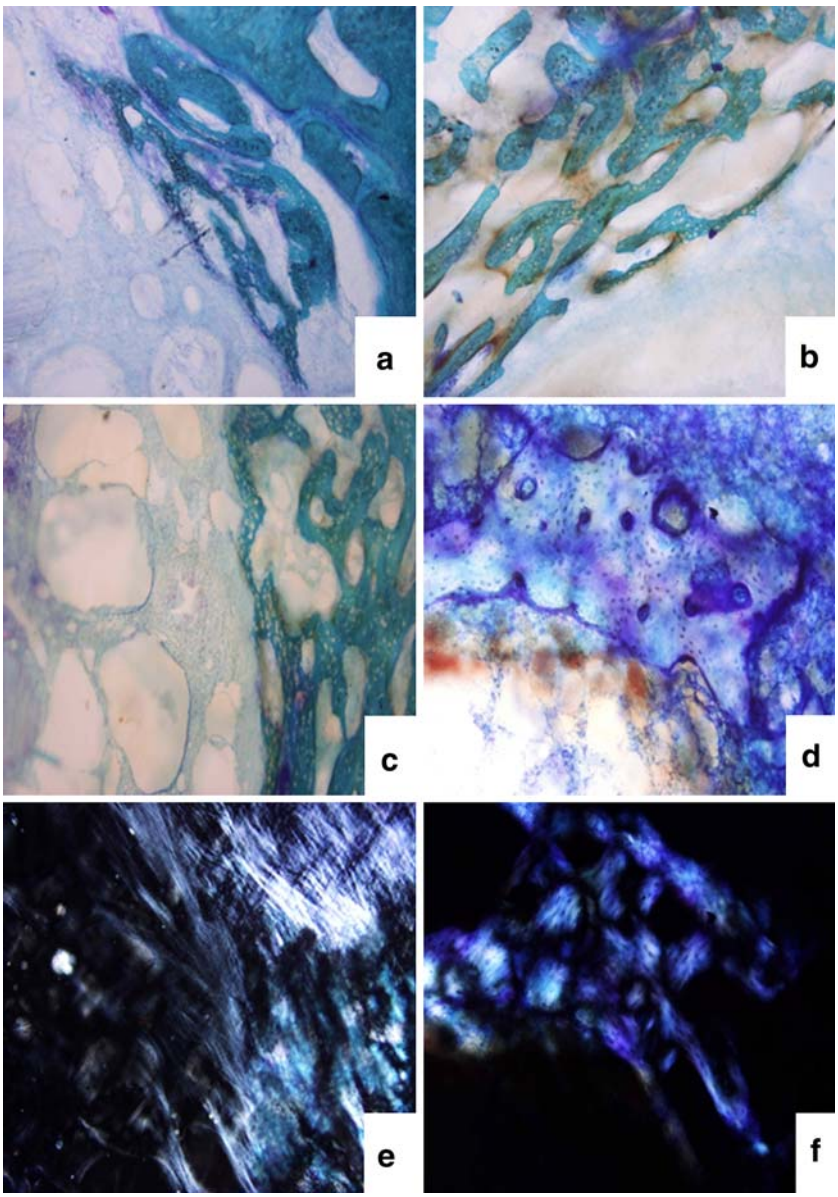

Fig. 3 Detailed histological features of bone repair in defects treated with SB bone filler (**a**, **c**, **e**) and Fisiograft® (**b**, **d**, **f**) after 4 (**a**, **b**) and 8 (**c**, **d**, **e**, **f**) weeks of implantation. Images show bone repair at the tissue/biomaterial interface. **a**–**d** LM analysis, **e**, **f** PLM analysis. Photos were taken at ×20 magnification

times (i.e. 4 and 8 weeks). Conversely, Fisiograft® gel completely degraded in all the cases regardless of bone regeneration that was unsatisfactory in two out three cases (Fig. 4d).

The histomorphometric (Table 3) and microhardness (Table 4) analyses showed that no significant differences were found in the percentage of bone repair and of bone in-growth in the external, medium and inner defect areas. Only in the case of the outer bone formation, the BFR and microhardness data after 24 weeks for the SB bone filler-treated defects were significantly higher than Fisiograft® gel ($P < 0.05$).

The preliminary assessment of the effect of SB packing on bone repair was performed by treating the femur defects with the alternative, three-component SB bone filler formulation. These experiments were performed in duplicate at week 8 and 16. Figure 5a, b shows that a gradual bone in-growth is qualitatively favored by the presence of relatively more spaced granules. The presence of spaced granules clearly encouraged the infiltration of osteoid with a progressively mineralizing front (Fig. 5b) and the alignment of osteoblasts on the granule surface still after 16 weeks (Fig. 5d, arrows). Blood vessels were also visible that suggest the occurrence of bone angiogenesis (Fig. 5c, asterisk).

4 Discussion

The present study highlights the bone regeneration performance of SB bone filler when compared to a

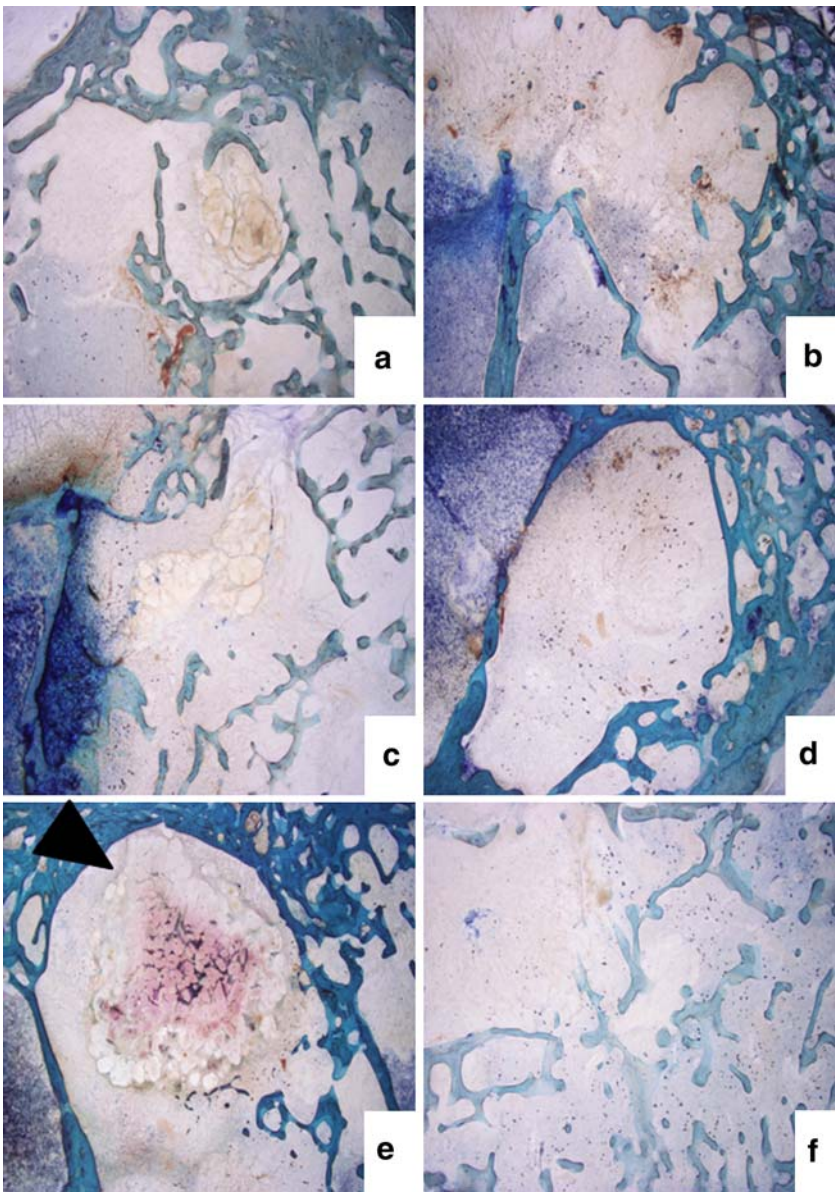

Fig. 4 Typical histological pattern of bone repair in defects treated with SB bone filler (**a**, **c**, **e**) and Fisiograft® (**b**, **d**, **f**) after 24 weeks of implantation. Images show a direct comparison (**a** vs. **b**, **c** vs. **d**, **e** vs. **f**) of the bone repair performance of the two biomaterials when implanted in contra-lateral femurs. Images were selected to show different degrees of bone in-growth and biomaterials packing and degradation. *Arrowhead* indicates the presence of fibrotic tissue. Photos were taken at 1.25× magnification

commercially available bone filler, the Fisiograft® gel. The ability of Fisiograft® gel and other PLA/PGA-based biomaterials to encourage bone formation in clinical applications is well known [28, 34]. The use of this type of biomaterial is widespread also in consideration of the ultimate safety of their degradation. However, although PLA/PGA biomaterials are completely degraded in CO_2 and water, the gradual breakdown of their polymeric chain is known to form relatively small molecular weight by-products triggering an inflammatory response [15–17]. Furthermore, the clinical performance of this type of biomaterial is associated to good osteoconductive properties. For this reason, the main use of Fisiograft® gel and other PLA/PGA biomaterials is mainly recommended for bone augmentation applications such as those required in periodontal applications. For the treatment of large critical size bone defects, autografts are still the material of choice and, only recently, recombinant human BMP-7 (rhBMP-7) combined with Type I bovine collagen (OP-1 Putty) and also carboxymethylcellulose (OP-1 Implant) from Styker Biotech (Hopkinton, MA, USA) have been made available to surgeons. These new bone fillers appear to be able to stimulate bone regeneration in experimental studies [35, 36] and in severe clinical cases [37, 38]. However, the presence of type I bovine collagen may still represent a risk for transmitting infectious diseases to humans such as bovine spongiform encephalopathy (BSE) or foot-and-mouth disease. For these reasons, in some countries the use of this biomaterial has been restricted by regulatory authorities [39, 40].

Table 3 Bone healing rate and bone formation expressed as percentage of three defined circular regions covering the total defect area or expressed as mineral apposition rate (MAR) or bone formation rate (BFR) in the outer region

	Experimental time (weeks)	Sample (*n*)	Bone healing rate (%)	Bone formation				
				% Outer region	% Middle region	% Inner region	MAR (μm/day)	BFR ($\mu m^2/\mu m/day$)
SB bone filler	4	6	27.6 (18.1–33.9)	43.9 (32.6–56.8)	6.9 (0.0–9.7)	0.0	–	–
	8	6	20.0 (12.2–21.6)	32.0 (28.7–38.1)	1.2 (0.0–3.1)	0.0 (0.0–0.9)	2.3 (1.9–2.8)	1.1 (0.8–1.3)
	24	5	19.2 (12.7–22.6)	31.4* (20.9–34.7)	3.3 (0.0–10.8)	0.0 (0.0–1.3)	1.6 (1.4–2.1)	1.1* (0.8–1.4)
Fisiograft® gel	4	6	26.9 (24.7–29.8)	47.6 (40.4–49.6)	6.4 (1.2–6.7)	0.0 (0.0–1.0)	–	–
	8	6	22.1 (11.1–39.2)	36.9 (20.0–58.5)	4.7 (0.0–19.9)	0.0 (0.0–6.4)	2.8 (1.1–3.4)	1.5 (0.5–2.5)
	24	5	17.2 (2.4–22.6)	28.7 (4.0–30.1)	4.5 (0.4–14.6)	4.1 (0.0–12.7)	1.3 (1.2–1.8)	0.6 (0.4–1.1)

Median (min–max)

Wilcoxon signed rank test: * SB bone filler versus Fisiograft® gel, $P < 0.05$

Table 4 Bone microhardness data on newly formed bone inside the defect at 4, 8 and 24 weeks

	Experimental time (weeks)	Sample (*n*)	$HV_{200\ \mu m}$	BMI
SB bone filler	4	6	–	–
	8	6	38.1 (29.5–49.5)	0.91 (0.72–1.26)
	24	5	40.5* (26.1–46.1)	0.87* (0.54–1.16)
Fisiograft® gel	4	6	36.1 (32.7–40.6)	0.94 (0.79–1.08)
	8	6	42.8 (36.6–46.3)	1.03 (0.80–1.15)
	24	5	37.5 (30.2–56.6)	1.01 (0.78–1.25)

Median (min–max)

Wilcoxon signed rank test: * SB bone filler versus Fisiograft® gel, $P < 0.05$

Recently, a novel class of biomaterial based on de-fatted soybean has been proposed with an ascertained induction on osteoblast differentiation and inhibition of macrophage activation and osteoclast tartrate-resistant acid phosphatase activity [25–27]. This material can be provided in form of membranes, blocks or granules as well as of hydrogels of different density. In this study, mixtures of granules and hydrogels at different ratios were prepared to provide different formulations that could be implanted either as a pre-packed injectable material or as a three-component kit including a gel reconstituting solution (0.1 M $CaCl_2$), a SB powder (to be reconstituted into hydrogel) and SB granules. The implantation procedure of the pre-packed material by SB bone filler was relatively easy to perform, but histological analysis clearly shows that it often led to an excessive packing of the granular material (Fig. 2a, c). The relatively limited exposed surface area in the excessively packed implants produced a relatively slow degradation of the biomaterial and, as a consequence, reduced bone infiltration. The inclusion of these specimens in the histomorphometrical and microhardness data analysis inevitably led to SB bone filler performances lower than Fisiograft® gel. Although these differences were shown to be significant by statistical analysis, they did not offer a direct comparison between the two biomaterials when implanted in the same animal in the two counter-lateral femurs. Indeed, a paired *t*-test of the score data was also considered to highlight the individual variability. Furthermore, the more accurate histological analysis of the biomaterial/tissue interface highlighted different patterns of bone repair for the two biomaterials. First of all, the study of the newly formed trabecular bone clearly indicated different mechanisms of action. The new bone infiltrating Fisiograft® gel-treated defects showed trabeculae with size and morphology not different from the non-damaged bone. Conversely, a dense network of relatively small trabeculae, known as reticular bone, was visible at week 4 and 8 of implantation of the SB bone fillers. This morphology is typical of enhanced bone activity that has been ascribed to the presence of isoflavones in the SB [26, 27]. Indeed, both purified soy isoflavones and SB have been shown to induce osteoblast differentiation and calcified bone *noduli* in vitro [26, 27]. In addition, the inhibitory effect of this plant estrogens on osteoclast activity has also been demonstrated [26, 27]. It has also been widely proven that soy isoflavones such as genistein and daidzein preferentially interact with the estrogen receptor beta of the nuclear membrane and, therefore, they are able to stimulate osteoblast differentiation without enhancing the risks of tumors as other known drugs used in hormone replacement therapy in osteoporotic women [26, 27]. Eventually, at longer implantation times, these small and dense trabeculae were

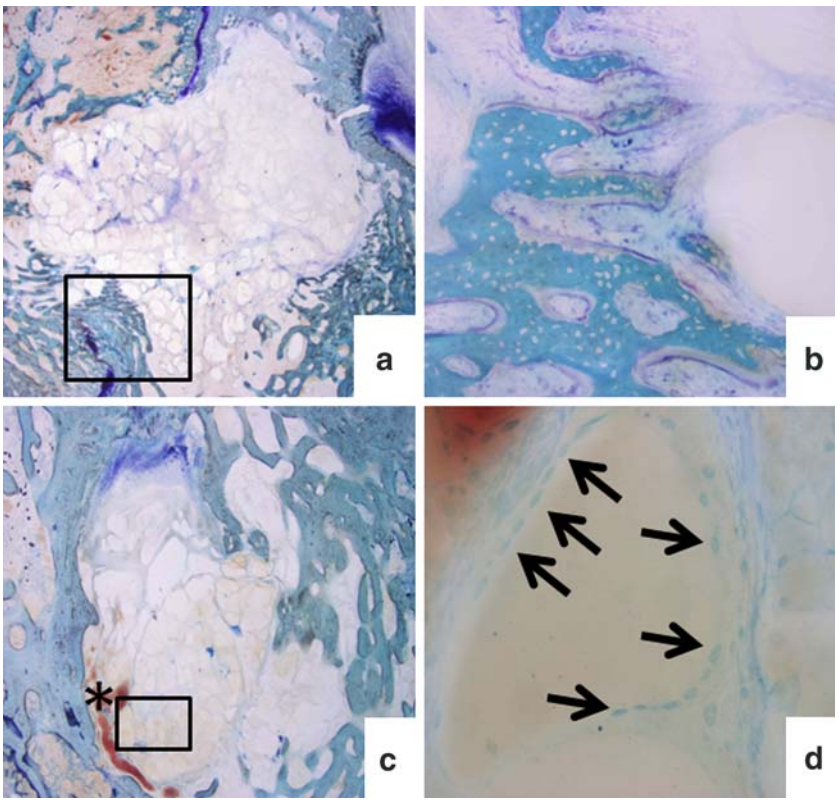

Fig. 5 Typical histological patterns of bone repair in defects treated with a 3-components SB bone filler after 8 (**a**, **b**) and 16 (**c**, **d**) weeks of implantation. Images show overall bone in-growth at 8 (**a**) and 16 (**c**) weeks and details of osteoid formation (**b**, front of non-mineralized collagen and mineralized tissue) and of osteoblast alignment (**d**) in close proximity of SB granules. *Asterisk* indicates blood vessels. *Arrows* indicate osteoblast alignment on a SB granule. Photos were taken at ×1.25 (**a**, **c**) and ×20 (**b**, **d**) magnification

remodeled into larger bone with morphology similar to that observed in the case of Fisiograft® gel-treated defects. More importantly, bone in-growth in SB bone filler-treated defects progressively developed over the 24 week period, while in the case of Fisiograft® gel clear indications of bone resorption were observed at week 8. This bone resorption was attributed to the pro-inflammatory activity of PLA/PGA fragments resulting from the biomaterial degradation [17]. An inflammatory reaction was also observed in the non-mineralized areas of SB bone filler-treated defects especially in their inner part. This reaction is likely to be due to the antigenic role exerted by the protein and carbohydrate components of soy. Although this did not lead to any bone resorption, it could lead to fibrotic capsule formation at longer term. However, histological features resembling fibrous tissue was found only in one of the 24-week implants and, in particular, it was found associated to an implant where the excessive granule packing led to a slow material degradation. The relatively large data variability may also be ascribed to different loading/gait patterns in each animal and between animals. Although no dedicated measurement was performed, the relatively narrow range of body weight of the used animals would rule out this effect on the data reproducibility.

Overall, the data collected in this study highlight that SB bone filler have a potential in stimulating bone regeneration in several clinical applications. As the adopted experimental model could not closely mimic the biomechanics of the typical orthopaedic applications, the SB fillers were compared to a commercial bone filler that is mainly used for periodontal applications, where the repair of trabecular bone is pursued. Despite its widespread use, Fisiograft® is not the only material used in such periodontal applications. For example, platelet-rich plasma, either as such or in combination with allograft, is preferred by many surgeons. Therefore, it would be interesting to compare the bone repair properties of the SB fillers also with these natural biomaterials. Such a comparison would be interesting in the light of the ascertained bioactivity of the SB on bone cells. Indeed, both the ability of the granules to support osteoblast alignment on their surfaces and the known bioactivity exerted by SB bone filler on these cells through the release of isoflavones are likely to stimulate bone repair. This seems to be confirmed by the short-term histological features of the newly formed bone that is characterized by a dense network of convolute and relatively small trabeculae. However, the SB bone filler formulation seems to be as important as its osteoconductivity and bioactivity properties. Indeed, only pastes preserving granule spacing upon implantation led to a satisfactory bone in-growth and degradation rate. Conversely, excessively packed or sparse granules failed to promote bone in-growth; the formulation with an excessive granule packing did not allow bone infiltration. This is a problem shared by any other biomaterial the degradation of which is relatively slow and not tuned with the rate of bone repair. Excessive material

packing is widely considered as a drawback in allograft and HA granules used in impaction grafting in applications such as total hip arthroplasty revision. However, the present study also showed that the formulations with a prevailing hydrogel component did not offer sufficient scaffolding, and maybe bioactive activity, to adequately support tissue repair.

5 Conclusions

Soybean-based biomaterials clearly promote bone repair through a mechanism of action that is likely to involve both the scaffolding role of the biomaterial for osteoblasts and the induction of cell differentiation. Therefore, these biomaterials have a potential to become fillers alternative to osteoconductive products such as those based on either autologous bone or ceramics or PLA/PGA hydrogels. Indeed, in addition to their bone repair potential, the ductility of SB bone filler biomaterials brings advantages in the surgical practice when compared to the brittle and not malleable ceramics or to the relatively loose consistency of hydrogels. However, their clinical performance of this new class of biomaterials will tightly depend on the optimization of their formulation.

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